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Evidence from Internally Transcribed Spacer Sequence Analysis of Soybean Strains that Extant *Bradyrhizobium* spp. Are Likely the Products of Reticulate Evolutionary Events[▽]

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The internally transcribed spacer (ITS) sequences of several members within each of 17 soybean bradyrhizobial serogroups were determined to establish whether the regions within all members of each serogroup were identical. The rationale was to provide a sequence-based alternative to serology. The objective also was to link the extensive older literature on soybean symbiosis based on serology with ITS sequence data for more recent isolates from both soybean and other legumes nodulated by rhizobia within the genus *Bradyrhizobium*. With the exception of serogroup 31 and 110 strains, sequence identity was established within each serogroup. Variation ranged from 0 to 23 nucleotides among serogroup 31 strains, and the regions in the type strains USDA 31 (serogroup 31) and USDA 130 (serogroup 130) were identical. Sequence identity was established among most strains within serogroup 110. The exceptions were USDA 452 and USDA 456, which had ITS sequences that were identical with those of the serotype 124 strain, USDA 124. Perhaps this would imply that USDA 452, USDA 456, and serogroup 31 strains are members of rhizobial lineages resulting from genetic exchange and homologous recombination events. This conclusion would be supported by the construction of a phylogenetic network from the ITS sequence alignment implying that the genomes of extant members of the genus *Bradyrhizobium* are likely the products of reticulate evolutionary events. A pairwise homoplasy index (phi or Φ_w) test was used to obtain further evidence for recombination. The ITS sequences of USDA 110 and USDA 124 were more divergent (53 nucleotides) than this region between the type strain *Bradyrhizobium japonicum* USDA 6^T and the proposed species *Bradyrhizobium yuanmingense* (28 nucleotides) and *Bradyrhizobium liaoningense* (48 nucleotides). Therefore, support for assigning discrete species boundaries among these three proposed species appears limited, considering the evidence for recombination, the narrow divergence of the ITS sequence, and their relative placement on the phylogenetic network.

The type strain for *Bradyrhizobium japonicum* (USDA 6^T), *B. japonicum* strain USDA 110, and the type strain for *Bradyrhizobium elkanii* (USDA 76^T), which are bacteria forming a nitrogen-fixing symbiosis with soybean, are often used as reference strains in the characterization of newly cultured rhizobia. USDA 110 has been included as a representative because the literature characterizing this soybean strain is the most extensive and because it was selected for whole-genome sequence analysis of *B. japonicum* (15). However, selecting only these three strains to represent *B. japonicum* and *B. elkanii* in comparative analyses with additional isolates of *Bradyrhizobium* disregards the full range of the diversity within these two rhizobial species (19). As a consequence, the evidence provided for grouping new isolates of *Bradyrhizobium* separately from *B. japonicum* and *B. elkanii* perhaps may have been incomplete. For example, the proposal of *Bradyrhizobium liaoningense* strain 2281 as a species separate from *B. japonicum* (26) did not include a comparison with *B. japonicum* strain USDA 135. This omission is significant because USDA 135 and strain 2281 harbored identical 16S rRNA genes with highly similar internally transcribed spacer (ITS) regions and shared serological determinants (19), evidence that perhaps would be inconsistent with the suggested classification of strain 2281.

The correlation between inoculation leading to formation of nodules on soybean roots and improved plant performance was well established as early as the late 1800s, long before this legume became an important crop in the United States (3, 7, 11). The value of inoculating soybean subsequently stimulated research that attempted to establish relationships between the rhizobia of soybean and other legumes. These early studies at first principally depended upon cultural characteristics and host plant infection (6, 13, 17) but also motivated development of serology for species identification as used with animal pathogens. As early as 1932 the existence of several serological groupings among soybean strains had been revealed (9), culminating in the description of 17 different serogroups by 1965 (8). Serology became an important approach for 50 years in soybean research, and the scientific literature on the serology of soybean strains is extensive.

With the more recent introduction of methodologies based on molecular biology, a significant problem to address is how to link the extensive literature on serology of the soybean rhizobia. An obvious approach would be to apply techniques of DNA analysis to members of the 17 serogroups in order to reveal potential differences. The soybean strains were sepa-

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rated into the two species *B. japonicum* and *B. elkanii* by DNA homology and standard restriction fragment length polymorphism analysis (16). Twelve of the serogroups were placed in the species *B. japonicum* and included USDA 6^T as the type strain and USDA 110, for which a complete genome sequence is available (15), while the remaining five serogroups were separated out into *B. elkanii*. Since differences in the 16S rRNA genes of these two species were limited (19), sequence variation of the ITS region was used to increase the resolution separating the 17 serotype strains (19). The assumption was that the DNA sequence of the ITS region is constant among members within the same serogroup. The identification of *B. liaoningense* strain 2281 as a member of *B. japonicum* serogroup 135 by both serology and ITS sequence provided some justification for this assumption. Subsequently, ITS region sequence divergence of soybean bradyrhizobia for which the serogroup affinity was unknown was compared with each of the 17 serotype strains (20). The ITS region also was used in a description of *Bradyhrizobium denitrificans*, where comparisons had been made with the 17 serogroup soybean strains (18, 21). However, in the initial description of *B. yuanmingense* (26) only USDA 6^T, USDA 110, and USDA 76^T were used, and only USDA 6^T was compared to strain 2281 in the initial description of *B. liaoningense* (25).

Before comparisons are made in future studies, it might be desirable first to determine whether soybean bradyrhizobia belonging to the same serogroup harbor ITS regions that are identical in DNA sequence. Since this has not been done, the objective was to determine the ITS sequences of several different members within each of the serogroups where available. The analysis led to further evidence that extant bradyrhizobial genomes are the result of reticulate evolutionary events.

MATERIALS AND METHODS

The USDA ARS National *Rhizobium* Germplasm Resource Collection maintains 156 cultures of soybean strains that have been analyzed for their serological affinity. Among these only one accession is available for serogroups 124, 126, and 130. In a preliminary analysis of these 156 strains, the ITS regions for each culture were amplified by PCR (19) with template DNA purified using a small-scale method (19) followed by restriction fragment length polymorphism analysis with MspI digests according to the method of Beyene et al. (2). Sequence analysis of the PCR products generated with selected strains for each serogroup (Table 1) was performed according to the method of van Berkum and Fuhrmann (19) and included several bradyrhizobia that had originated from different host legumes including USDA 3051 from *Lupinus angustifolius* (GenBank accession no. EU834723), USDA 3470 from *Lotus uliginosus* (GenBank accession no. EU834724), USDA 3384 from *Crotolaria paulina* (GenBank accession no. EU834728), USDA 3456 from *Vigna unguiculata* (GenBank accession no. EU834727), and USDA 3426 and USDA 3259 from *Phaseolus lunatus* (GenBank accession no. EU834722 and EU834721, respectively). The ITS sequences were imported into and then were aligned with GeneDoc version 2.6.001 (K. B. Nicholas and H. B. Nicholas [http://www.nrbsc.org/gfx/genedoc/index.html]). Five additional ITS region sequences, for *B. yuanmingense* (GenBank accession no. AJ534605, AY386734, AY599094, and AY599095) and *B. liaoningense* (GenBank accession no. AF208513), were included in the analysis. The alignment was exported as a fasta file and subsequently converted to a Nexus file and imported into SplitsTree version 4.8 (12) to display the phylogeny of the bradyrhizobial ITS region as a NeighborNet (5). NeighborNet is a linkage tree algorithm similar to neighbor joining or the unweighted pair group method using average linkages, but pairing and combining of nodes are different to take into consideration the possibility that there may be alternate evolutionary histories resulting from gene transfer and recombination. The USDA 4967 strain of *B. denitrificans* (21) was used as the outgroup, and confidence values were calculated by bootstrap analysis using 500 permutations of the data set. Confidence values of 95% and above were indicated on the diagram. The software PhiPack was used to test for recombination within the *Bradyrhizobium* ITS region using the pairwise homoplasy index (Φ_w statistic) of Bruen et al. (4) (http://www.mcb.mcgill.ca/~trevor/). This method is less prone to falsely infer recombination when levels of recurrent mutation are high and measures the significance of the phylogenetic discrepancy across the alignment. The test is based on the compat-

TABLE 1. Serotype and serogroup strains used in sequence analysis of the 16S rRNA-23S rRNA ITS region[a]

Serogroup	USDA strain accession	Date and location of isolation	GenBank accession no.[b]
4	**USDA 4**	1932, Iowa	AF208515
	USDA 51	1932, Virginia	
	USDA 54	1936, Maryland	
6	**USDA 6**	1929, Japan	U69638
	USDA 41	1929, Japan	
	USDA 50	1931, Japan	
31	USDA 26	1940, North Carolina	EU834734
	USDA 29	1936, unknown	EU834730
	USDA 31	1941, Wisconsin	AF208512
	USDA 33	1926, unknown	EU834729
	USDA 39	1929, Japan	EU834737
	USDA 40	1941, New Jersey	EU834732
	USDA 61	1946, North Carolina	EU834736
	USDA 67	1948, North Carolina	EU834738
	USDA 83	1956, Maryland	EU834735
	USDA 116	1959, Brazil	EU834733
	USDA 120	1956, Illinois	EU834731
38	**USDA 38**	1929, Japan	AF208514
	USDA 45	1929, Japan	
	USDA 56	1946, North Carolina	
46	**USDA 46**	1943, Alabama	AF208516
	USDA 71	1948, Arizona	
	USDA 100	1957, unknown	
62	**USDA 62**	1946, North Carolina	AF208517
	USDA 140	1959, Bolivia	
76	**USDA 76**	1956, plant passage of USDA 8	U35000
	USDA 77	1956, plant passage of USDA 8	
	USDA 103	1953, Mississippi	
	USDA 117	1959, Mississippi	
94	USDA 93	1956, North Carolina	
	USDA 94	1956, North Carolina	AF208518
	USDA 99	1956, North Carolina	
	USDA 119	1969, South Carolina	
110	USDA 17	1955, Yugoslavia	
	USDA 110	1959, Florida	Z35330
	USDA 137	1961, Iowa	
	USDA 443	1989, Arkansas	
	USDA 445	1989, plant passage of USDA 110	
	USDA 452	1989, Wisconsin	EU834725
	USDA 456	1989, Wisconsin	EU834726
122	**USDA 122**	1960, Mississippi	AF208503
	USDA 132	1948, Louisiana	
	USDA 136	1961, Maryland	
123	**USDA 123**	1960, Iowa	AF208504
	USDA 432	1989, Minnesota	
	USDA 438	1989, South Dakota	
124	**USDA 124**	1960, Mississippi	AF208505
126	**USDA 126**	1961, Maryland	AF208507
127	**USDA 127**	1961, Iowa	AF208508
	USDA 424	1989, Delaware	
	USDA 430	1989, Minnesota	
129	**USDA 129**	1961, Iowa	AF208509
	USDA 422	1989, Arkansas	
	USDA 427	1989, Iowa	
130	**USDA 130**	1961, Maryland	AF208510
135	**USDA 135**	1961, Iowa	AF208511
	USDA 479	1989, Louisiana	
	USDA 489	1989, South Dakota	
	USDA 490	1989, South Dakota	

[a] The serotype strains are in bold and define the serology of the rhizobia of soybean.

[b] The absence of a GenBank accession number for serogroups 4, 6, 38, 46, 62, 76, 94, 122, 123, 127, 129, and 135 indicates that the ITS sequence for the entry was identical to the sequence obtained with the serotype strain. GenBank accession numbers for ITS region sequences of other USDA accessions in the collection are indicated in the text.

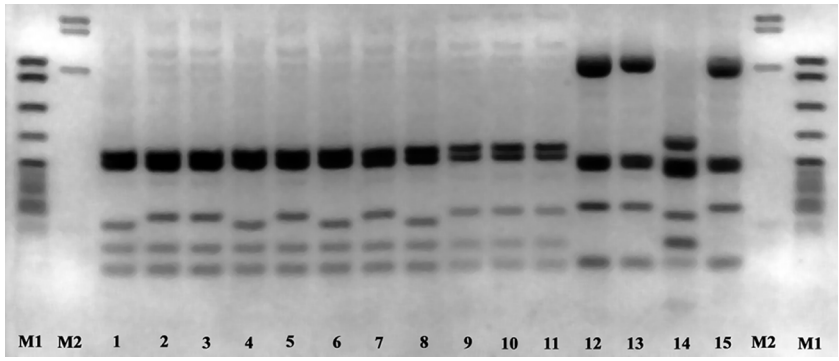

FIG. 1. Electrophoretic mobilities of DNA fragments resulting from MspI digests of PCR products generated with serogroup 31 and 110 strains using primers for amplification of bradyrhizobial ITS regions between the 16S and 23S rRNA genes. Lanes: M1, pBR322 MspI digest; M2, lambda EcoRI HindIII digest; 1, USDA 26; 2, USDA 29; 3, USDA 31; 4, USDA 33; 5, USDA 39; 6, USDA 61; 7, USDA 67; 8, USDA 83; 9, USDA 120; 10, USDA 116; 11, USDA 40; 12, USDA 452; 13, USDA 456; 14, USDA 110; 15, USDA 124.

ibility of informative sites providing a P value, which when significant ($P > 0.05$) would indicate that events of recombination are highly likely.

The serological affinities of the selected cultures were confirmed by enzyme-linked immunosorbent assay (10) using heat-treated whole cells and polyclonal antisera produced in rabbits against the serotype strains (24). Cells were cultured in 4 ml yeast extract mannitol broth (25) for 7 to 10 days at 28°C, steamed at 100°C for 20 min, and diluted with 0.85% NaCl to an A_{600} of approximately 0.2. The enzyme-linked immunosorbent assay was done as described by Fuhrmann and Wollum (10), and the final absorbance of each well was measured at 405 nm using an automated microplate reader (model ELx800; Bio-Tek Instruments).

RESULTS AND DISCUSSION

The fingerprint patterns within each of the serogroups were identical (data not shown) with the exception of serogroups 31 and 110 (Fig. 1). The fingerprint patterns obtained with the serotype 31 strain USDA 31 and strains USDA 29, USDA 39, USDA 40, USDA 67, USDA 116, and USDA 120 were identical, while a different pattern was obtained with USDA 26, USDA 33, USDA 61, and USDA 83 (Fig. 1). The fingerprint patterns detected with the serotype 110 strain USDA 110 and USDA 452 and USDA 456 were different. However, the fingerprint patterns of these two serogroup 110 strains and the serotype strain for serogroup 124, USDA 124, were identical (Fig. 1).

With the exception of serogroup 31 and 110 strains, sequence identity was established within each serogroup (Table 1). These included strains of serogroup 4 (GenBank accession no. AF208515), serogroup 6 (GenBank accession no. U69638), serogroup 38 (GenBank accession no. AF208514), serogroup 46 (GenBank accession no. AF208516), serogroup 62 (GenBank accession no. AF208517), serogroup 76 (GenBank accession no. U35000), serogroup 94 (GenBank accession no. AF208518), serogroup 122 (GenBank accession no. AF208503), serogroup 123 (GenBank accession no. AF208504), serogroup 127 (GenBank accession no. AF208508), serogroup 129 (GenBank accession no. AF208509), and serogroup 135 (GenBank accession no. AF208511). Additional members within serogroups 124, 126, and 130 were not available.

None of the soybean strains had ITS sequences that were identical with those of rhizobia that originated from other legume hosts including USDA 3051 from *Lupinus angustifolius*, USDA 3470 from *Lotus uliginosus*, USDA 3384 from *Crotolaria paulina*, USDA 3456 from *Vigna unguiculata*, USDA 3426 and USDA 3259 from *Phaseolus lunatus*, and the published sequences of *B. liaoningense* (GenBank accession no. AF208513) strain 2281 (26) and *B. yuanmingense* (GenBank accession no. AJ534605, AY386734, AY599095, and AY599094) with CCBAU 10071^T as the proposed type strain (27). With the exception of *B. denitrificans*, the levels of the sequence divergence across these bradyrhizobia were similar, ranging from 0 to 103 nucleotide substitutions in 1,162 sites. The ITS regions of the soybean strain USDA 38 and the two strains from *P. lunatus*, USDA 3259 and USDA 3426, were the most distant (103 and 102 nucleotides, respectively). The ITS regions between *B. japonicum* USDA 135 and *B. liaoningense* 2281 (USDA 3622) differed by two nucleotides. Across bradyrhizobia from soybean and other legume hosts the ITS sequences of the soybean strain USDA 127 and strain USDA 3470 from *L. uliginosus* were the most similar, with 13 nucleotide substitutions. The ITS region of *B. japonicum* USDA 6^T and that of *B. yuanmingense* CCBAU 10071^T varied by 37 nucleotide substitutions, while with *B. japonicum* USDA 110 and USDA 124 the differences were 46 and 29 nucleotide substitutions, respectively. By comparison, the most distant ITS regions within *B. japonicum* were between USDA 124 and USDA 123 or USDA 135 with 61 nucleotide substitutions. Of 66 comparisons among the serotype strains of *B. japonicum*, 39 had ITS sequences that were more distant than or equal to that between the type strains for *B. japonicum* and *B. yuanmingense*.

Sequence divergence was evident within the ITS region among serogroup 31 strains and between the serogroup 110 strains and USDA 452 and USDA 456. The most distant ITS sequence within serogroup 31 strains was 23 nucleotide substitutions. The ITS sequences of the serotype strain for serogroup 130, USDA 130 (GenBank accession no. AF208511), and of serogroup 31 strains USDA 29, USDA 31, USDA 39, USDA 40, USDA 67, USDA 116, and USDA 120 were identical. The ITS sequences of strains USDA 26, USDA 61, and USDA 83 were identical and varied from that of USDA 31 by seven nucleotide substitutions. The serological affinities of USDA 26, USDA 29, USDA 33, USDA 39, USDA 40, USDA 61, USDA 67, USDA 83, USDA 116, and USDA 120 with serum prepared against USDA 31 were confirmed. Variation among rhizobia of serogroup 31 has not been reported before. Evidently, bradyrhizobia grouped by their serological affinity

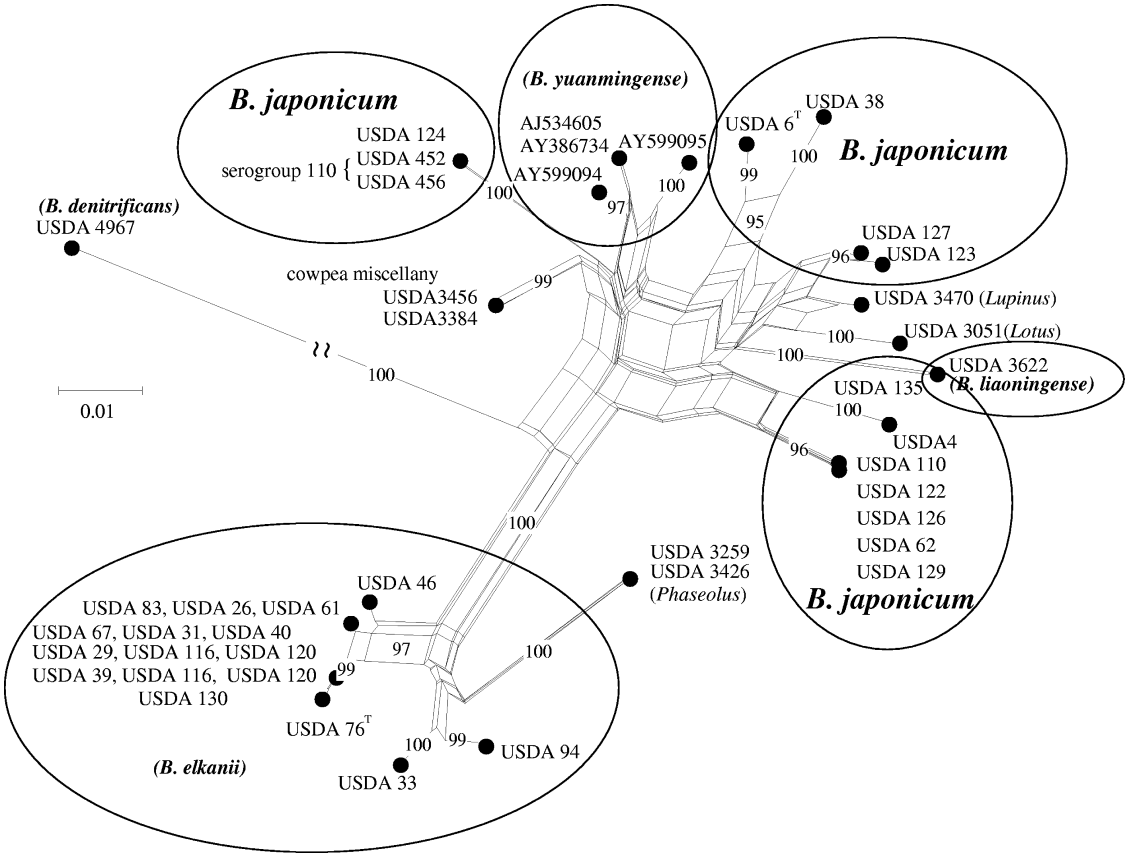

FIG. 2. Phylogeny of the bradyrhizobial ITS region generated from sequences aligned with GeneDoc version 2.6.001 (K. B. Nicholas and H. B. Nicholas [http://www.nrbsc.org/gfx/genedoc/index.html]) and portrayed as a NeighborNet (5) with SplitsTree version 4.8 (12). GenBank accession numbers are indicated for the four sequences that are available for *B. yuanmingense*. The GenBank accession number for the ITS sequence of *B. liaoningense* was AF208513.

may harbor divergent ITS regions. Also, members belonging to different serogroups may harbor identical ITS sequences.

Within serogroup 110 the ITS sequences were identical with that of the serotype strain USDA 110 (GenBank accession no. Z35330), except for strains USDA 452 and USDA 456, which were identical with the serotype strain for serogroup 124, USDA 124, GenBank accession no. AF208505 (Fig. 2). Both USDA 452 and USDA 456 cross-reacted with antisera prepared against USDA 110, thus confirming their placement within serogroup 110. The atypical result obtained with USDA 452 and USDA 456 is further confirmation that these two strains are uncharacteristic of serogroup 110. The strain USDA 456 was reported to have very specific phage susceptibility among 34 serogroup 110 strains, while USDA 452 was not susceptible when challenged with phage prepared against three different serogroup 110 strains (1). Both these strains lacked homology with cloned uptake hydrogenase genes, unlike an additional 13 Hup^--phenotype serogroup 110 strains that yielded positive signals (22). Also, these two strains fall into a distinctive group on the basis of *nod* probe hybridization analysis (22). Lohrke et al. (14) reported that these two strains were placed into one of two very similar groups based on electrophoretic mobilities of enzyme loci but unlike other members within their group were not restricted for nodulation on soybean PI 417566. The strain USDA 124 was not included in the study by Lohrke et al. (14); enzyme mobility and nodulation data for this strain are not available.

Evidence that genomes of extant members of the genus *Bradyrhizobium* are likely the products of reticulate evolutionary events was first reported based on an analysis of the 16S rRNA genes (23). The data obtained here with the ITS region further support this conclusion because of a significant result with the pairwise homoplasy index (4) where $P = 5.0 \times E^{-12}$ and because a phylogenetic network was produced (12) from the aligned sequences (Fig. 2). Phenotypic evidence for recombination was obtained from serology, where different members within serogroups 31 and 110 harbored ITS sequences that were variable and were observed in strains belonging to different serogroups. Possibly the data obtained with USDA 452 and USDA 456 may indicate that these two strains are members of rhizobial lineages that were the result of genetic exchange and homologous recombination events between serogroups 110 and 124. Similarly, extant serogroup 31 strains could be the result of a reticulate evolutionary history, since different ITS sequences are associated with the same serogroup. Considering the limited and overlapping divergence in the ITS regions between strains of *B. japonicum* with *B. yuanmingense* and *B. liaoningense* as well as the evidence that extant members within the genus *Bradyrhizobium* perhaps are descendants from a reticulate evolutionary history, it may be inappropriate to par-

tition these three groups and assign discrete species boundaries between them.

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